

# A Feasibility Study on the Identification of Potential Biomarkers in Pulmonary Embolism Using Proteomic Analysis

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## Abstract

Acute pulmonary embolism (PE) is a common emergency with a high morbidity and mortality. Most clinical presentations are non-specific and there is a lack of suitable biomarkers for PE. For example, the traditional D-dimer tests shows a rather high sensitivity for PE, but yet a rather low positive predictive value due to its lack of specificity. Research on novel biomarkers for PE is thus of interest to improve early diagnostics and reduce the number of unnecessary computed tomography pulmonary angiogram (CTPA) scans performed. In this study we evaluate the feasibility to use label-free quantitative proteomics to discover potential biomarkers for acute PE and to monitor changes in proteins levels in PE patients over time. Blood was collected from 8 patients with CTPA verified PE and from 8 patients presenting with same symptoms but with a negative CTPA. The samples were analyzed by liquid chromatography-mass spectrometry and thirteen protein concentrations were found to be significantly changed in PE patients compared to the CTPA negative controls. This exploratory study shows that proteomic analysis can be used to identify potential biomarkers for PE as well as to monitor changes of protein levels over time.

The complement proteins play a part in PE but further studies are needed to clarify their specific role in the pathophysiological process and to look for more specific proteins.

## Keywords

pulmonary embolism, biomarker, proteomics, complement factors, venous thromboembolism

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## Introduction

Acute pulmonary embolism (PE) is a common emergency and has a high morbidity and mortality.<sup>1</sup> In Sweden the incidence is estimated to be 60/100 000/year and it is increasing with age.<sup>2</sup> The clinical presentations are non-specific and the disease often mimic other common diagnoses found in an emergency department (ED).<sup>3</sup>

Current laboratory tests and biomarkers are not diagnostic for PE but can be used together with decision rules, such as the PERC rule or WELLS score, to alter the clinical suspicion and in this way help physicians in their diagnostic work.<sup>4,5</sup> D-dimer tests show a high sensitivity but low specificity for PE. Computed tomography pulmonary angiography (CTPA) is considered as the gold standard in diagnosis of PE, but there is a growing concern related to the radiation risks.<sup>6,7</sup> To improve early diagnostics and reduce unnecessary CTPA,

novel biomarkers are thus called for. To maximize their usability, these biomarkers need to fulfill at least some of the criteria for an ideal biomarker. For example, the biomarkers should be visible at an early stage, prior to histopathological changes, and be indicative after active damage. Additionally, they should be sensitive, but also correlate with the severity of damage.<sup>8</sup> Furthermore, biomarker selection should be driven by an

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attempt to answer an explicit clinical question, preferably a causative relationship of the biomarker to disease-state.<sup>9</sup>

Earlier research regarding new biomarkers in venous thromboembolism (VTE), including PE, has often used blood samples from the post-acute phase when the patient has been started on treatment with anticoagulants. Alternatively, the samples have included patients with deep venous thrombosis (DVT) and PE.<sup>10</sup> In the present study we evaluate the feasibility to use proteomic analysis to find suitable, new biomarkers in patients with acute PE. Screening for new biomarkers by a proteomic approach allows a simultaneous detection of changes in multiple proteins in each study.<sup>11</sup> It is also a suitable way to monitor changes in protein expression over time. The use of proteomics in this area thereby opens up the possibility to find entirely new biomarkers that traditionally has not been known to have any part in thrombosis or coagulation.

## Methods and Patients

### Patients

A total of 16 male patients participated in this study performed at a County Hospital in Sweden, where the ED has about 45 000 annual visits. The patients were all non-smokers without malignancy, and a strictly male patient group was chosen to avoid patients with oral contraceptives and hormone treatment, factors that may affect coagulation and the risk of VTE. Eight of the patients had CTPA verified PE at their visit to the ED and were thus included in the study group. The other eight patients presented similar symptoms as the patients in the study group upon arrival at the ED, but had a negative CTPA and were thereby assigned to a control group. In addition to the initial symptoms, the patients in the study group and the control group were also matched according to cardiovascular risk factors and were all of the same ethnic origin and with similar socio-economic background. The initial symptoms were mainly related to acute onset of dyspnea, cough, chest pain during inspiration and chest pain during rest. Data on current vital signs and routine laboratory tests was gathered from the hospitals electronic medical record, and the basic characteristics of the 16 patients, including age, vital signs, ECG rhythm, D-dimer, Troponin T (TnT) and C-reactive protein (CRP) levels are given in Table 1.

The study was approved by the regional ethics committee, and informed written consent was obtained from all the participants before inclusion.

### Blood Sampling

Peripheral venous blood samples were taken at the ED from the patients in both groups after CTPA, but before any initiation of anticoagulation treatment. The samples were centrifuged within 30 min at 2000 *g* for 20 min and aliquots of citrated plasma were frozen and stored at  $-70^{\circ}\text{C}$  pending analysis. A follow up blood sample from the patients in the study group was taken at least 12 months after the first one. The same protocols

for sampling, sample handling and analysis were applied for these samples.

### Sample Preparation

**Depletion.** Multiple Affinity Removal System (MARS Hu-14) cartridge was used for depletion of the samples. Prior to depletion, an aliquot of 10  $\mu\text{L}$  of each plasma sample was diluted up to 200  $\mu\text{L}$  with buffer A (Agilent Technologies, Palo Alto, CA, USA). The diluted samples were filtered through 0.22  $\mu\text{m}$  cellulose acetate spin filters (Agilent Technologies) by centrifugation at  $15000\times g$  for 2 min. Afterwards, an aliquot of 190  $\mu\text{L}$  filtered and diluted plasma sample was loaded onto MARS Hu-14 cartridge and the flow-through (FT) fraction was collected by centrifugation for 2 min at  $100\times g$ . Two successive wash steps with 400  $\mu\text{L}$  of Buffer A were carried out to obtain maximum yield. The FT and wash (W) fractions were combined. The spin cartridge was washed with 2 ml of Buffer B (Agilent Technologies) to remove bound proteins and was then re-equilibrated with Buffer A. The remaining fractions (FT + W) were dried using a speed vac.

**Digestion.** The entire amount of protein in each depleted plasma sample was digested with trypsin. Briefly, the proteins were denaturated in 50  $\mu\text{L}$  of digestion buffer (6 M urea, 50 mM ammonium bicarbonate [AmBi]) and sonicated in a sonication bath for 1 min. Afterwards, a volume of 50  $\mu\text{L}$  of 50 mM AmBi was added to all samples. The proteins were reduced, alkylated and on-filter digested by trypsin according to a standard operating procedure. The collected peptide filtrate was vacuum centrifuged to dryness using a speedvac system. Dried peptides were redissolved in 100  $\mu\text{L}$  of 0.1% formic acid and further diluted 6 times prior to nano-LC-MS/MS.

### LC-MS/MS Analysis

The peptides were separated in reversed-phase on a C18-column with 90 min gradient and electrosprayed on-line to a Q Exactive Plus mass spectrometer (Thermo Finnigan). Tandem mass spectrometry was performed applying HCD.

### Data Analysis

The 24 RAW-data LC-MS/MS files obtained were quantitatively analysed by the quantification software MaxQuant 1.5.1.2. Proteins were identified by searching towards proteins extracted from Uniprot in November 2018. The search parameters were set to Taxonomy: *Homo sapiens*, Enzyme: Trypsin, Fixed modification: Carbamidomethyl (C), and Variable modifications: Oxidation (M), Deamidated (NQ). The search criteria for protein identification were set to at least two matching peptides of 95% confidence level per protein.

The results were exported into MS Excel (Microsoft, Redmond, WA, USA) for manual data interpretation and statistical analysis. The most likely biomarkers in the data set were

**Table 1.** Basic characteristics of patients in the study (S) and control (C) groups upon arrival to the emergency department.

Patient ID	Respiratory frequency	Saturation (Air)	Heart rate	ECG rhythm	D-dimer	CRP	TnT
S1	16	97	80	Sinus	1.6	5	20
S2	14	96	80	Sinus	0.7	45	<5
S3	28	90	110	Sinus	4.8	17	65
S4	15	99	100	Sinus	0.8	23	-
S5	16	100	56	Sinus	5.4	3	8
S6	16	99	72	Sinus	13	40	16
S7	28	97	94	Sinus	5.6	6	10
S8	26	73	120	Sinus	5.6	78	33
C1	17	98	94	Sinus	<0.2	2	9
C2	18	100	80	Sinus	<0.2	<0.6	<5
C3	16	97	75	Sinus	0.5	14	<5
C4	16	99	92	Sinus	0.7	5	-
C5	20	96	89	Sinus	0.8	5	7
C6	16	98	68	Sinus	-	19	<5
C7	16	99	82	Sinus	1.7	<0.6	-
C8	20	97	70	Sinus	1.3	4	20

pin-pointed by a search rule stating that the protein had to be identified in all 24 samples, that it should show a significant difference ( $p < 0.05$  according to Welch's unequal variances t-test) in comparison between study group and control, and also a significant change ( $p < 0.05$  according to paired t-test) back towards the control group level in comparison between acute and follow-up samples from the study group.

For those proteins fulfilling the above mentioned criteria, a principal component analysis (PCA) was performed in MATLAB R2018a (The MathWorks Inc., Natick, MA) to further illustrate the differences between the samples.

## Results

In total, 430 different proteins were tentatively identified in the data set. Of these, 242 were recovered in quantifiable amounts in more than one of the samples and 137 were present in all samples. Nineteen of these 137 plasma proteins showed a significant difference in concentration between patients with PE and matched controls in the acute setting. All of these proteins tended to be restored towards the control group level for the follow-up samples, and significantly so for the 13 proteins listed in Table 2. Three of these proteins were increased and ten were decreased in PE patients compared to controls.

Several of the proteins found, and described below, are complement proteins and part of a complex system of multiple proteins acting in inflammation and hemostasis and these are non-specific for a certain disease.<sup>12</sup>

### Proteins with an Increase in PE Compared with Controls

Complement component 9 was one of the proteins that showed an increased concentration for the study group. It is a protein that binds to the assembling membrane attack complex (MAC).<sup>13</sup> The interaction between the complement system and hemostatic factors maintain hemostasis under physiologic conditions and promote thrombosis under the pathologic

conditions as manifested in thrombotic complications of paroxysmal nocturnal hemoglobinuria and atypical hemolytic syndrome.<sup>14,15</sup>

Another protein that increased in concentration for the PE patients was Complement factor H, which is also a part of the complex complement system. Abnormalities of Complement factor H are associated with thrombolytic thrombocytopenic purpura, hemolytic uremic syndrome and thrombotic microangiopathy.<sup>16,17</sup>

The final protein with a marked increase for the study group compared to the control was Leucine-rich alpha-2-glycoprotein, which is a type 1 acute phase protein whose expression is up-regulated by the mediators of acute-phase response.<sup>18</sup> It has previously been shown to be involved in angiogenesis.<sup>19</sup>

### Proteins with a Decrease in PE Compared with Controls

Among the proteins that showed decreased concentrations for the study group, Antithrombin III (AT III), Coagulation factor XII and Carboxypeptidase B2 have a known part in the pathways regulating hemostasis.<sup>20-22</sup>

AT III is a nonvitamin K-dependent protease that inhibits coagulation by neutralizing the enzymatic activity of thrombin (factors IIa, IXa, Xa).<sup>23</sup> Patients with inherited AT III deficiency have a significantly increased risk of VTE and are also known to be resistant to anticoagulation by heparin.<sup>24</sup>

Studies on Coagulation factor XII show that it may contribute to the extent of developing thrombus in the intravascular compartment but also that it has a role in injury repair and angiogenesis.<sup>25</sup>

Activated Carboxypeptidase B2 reduces fibrinolysis and the potential use of carboxypeptidase inhibitors as a new drug treating VTE is interesting.<sup>26</sup>

Several proteins found, and described below, are not known for any specific regulatory role in thrombosis and haemostasis.

**Table 2.** Label free quantitative (LFQ) data and p-values for the 13 plasma proteins identified as potential biomarkers for pulmonary embolism. The LFQ values represent signal averages for the control group (C) and the study group in the acute (SA) and follow-up samples (SF), while the p-values are obtained from comparisons between the groups with t-tests.

Protein	ID	LFQ C	LFQ SA	LFQ SF	p-value SA versus C	p-value SA versus SF
<b>Increasing proteins</b>						
Complement component 9	A0A024R035	0.7E10	1.1E10	0.6E10	0.004	0.003
Complement factor H	B2RA39	2.7E8	4.4E8	2.7E8	0.027	0.033
Leucine-rich $\alpha$ -2-glycoprotein	P02750	2.7E9	3.9E9	1.9E9	0.047	0.019
<b>Decreasing proteins</b>						
Apolipoprotein C-III	A3KPE2	1.5E9	0.8E9	1.9E9	0.047	0.035
Carboxylic ester hydrolase	P06776	6.6E8	4.0E8	5.7E8	0.027	0.037
Antithrombin-III	A0A024R944	4.8E10	3.1E10	4.4E10	0.002	0.018
Procollagen C-endopeptidase enhancer	Q15113	7.0E7	4.6E7	6.9E7	0.016	0.024
Serpin peptidase inhibitor, clade A, member 4	B2R815	2.4E9	1.7E9	3.0E9	0.030	0.000
Carboxypeptidase B2	A0A087WSY5	1.2E9	0.8E9	1.1E9	0.021	0.032
Afamin	P43652	1.7E10	1.2E10	1.7E10	0.020	0.012
Serpin peptidase inhibitor, clade A, member 5	A0A024R6N9	5.2E8	3.9E8	6.6E8	0.018	0.001
Coagulation factor XII	P00748	5.7E9	4.4E9	6.1E9	0.024	0.012
N-acetylmuramoyl-L-alanine amidase	Q96PD5	2.5E9	2.1E9	3.1E9	0.018	0.000

Apolipoprotein C-III is secreted by the liver and small intestines and overexpression in humans contribute to atherosclerosis.<sup>27</sup>

Carboxylic ester hydrolase catalyze the hydrolysis of ester bonds into alcohols and carboxylic acids.<sup>28</sup>

Procollagen C-endopeptidase enhancer mediates HDL function by reducing lipid and immune cell accumulation in the artery.<sup>29</sup>

Serpin peptidase inhibitors are part of a family of protease inhibitors. They are central in controlling many proteolytic cascades, including the mammalian coagulation pathways.<sup>30</sup>

Afamin is a plasma vitamin E-binding glycoprotein primarily expressed in liver and has been found strongly associated with all components of metabolic syndrome.<sup>31</sup>

N-acetylmuramoyl-L-alanine amidase belongs to a family of hydrolases and proteomic research has suggested that it has a role in differentiating between infectious and non-infectious inflammatory syndromes.<sup>32</sup>

## Discussion

Shortness of breath and chest pain are two very common symptoms for patients presenting in the ED. For the physician it is imperative to at an early stage be able to differentiate and diagnose if these symptoms are due to a myocardial infarction or PE or any other less deadly diagnosis.

An overuse of computed tomography leads to immediate effects with regards to crowding and longer turn over times in an ED.<sup>33</sup> And it may also lead to an increase in cancer in otherwise healthy individuals.<sup>34</sup>

There are currently no reliable biomarkers for early diagnosis of PE. D-dimer can in some cases be used together with a validated scoring system to dismiss the PE diagnosis but an elevated D-dimer test can be found in several other conditions.

As seen in the results many biomarkers are part of the complex systems relating to acute and chronic inflammation as well as coagulation. Numerous previous studies have shown that PE activates coagulation factors as well as inflammatory factors.<sup>35</sup> This study unfortunately do not provide final evidence to what comes first, thrombosis or inflammation.

There are several limitations to this study. The number of patients is quite low and the study should be seen as a basis for further research with larger sample size. The fact that it is all male reduces its generalizability to the population as a whole.

In conclusion, this exploratory study shows that it is feasible to use proteomic analysis to look for potential biomarkers in this specific disease and that the complement system and its proteins play a part in acute PE. An interesting observation is that many of the proteins increased or decreased in this study play a part in general inflammation and atherosclerosis as well as in hemostasis and thrombus formation. It is therefore possible that the results can be used for further investigations relating to biomarkers in the metabolic syndrome and related diseases.

The findings are currently not clinically applicable and the current method of screening is too slow to influence clinical interventions. However, the results can be used as a basis for future research. Further studies are needed to clarify the proteins' specific role in the pathophysiological process and to look for more specific proteins outside the complement system as well. Additional studies may also clarify the possible role of the proteins from this study in risk stratification, development of prediction rules and treatment strategies with regards to PE.

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